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Angiotensin-(1-12) in the rostral ventrolateral medullary pressor area of the rat elicits sympathoexcitatory responses

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Abstract

The rostral ventrolateral medullary pressor area (RVLM) is known to be critical in the regulation of cardiovascular function. In this study, it was hypothesized that the RVLM may be one of the sites of cardiovascular actions of a new angiotensin, angiotensin-(1-12) [ANG-(1-12)]. Experiments were carried out in urethane-anaesthetized, artificially ventilated, adult male Wistar rats. RVLM was identified by microinjections of L-glutamate (5 mM). The volume of all microinjections into the RVLM was 100 nl. Microinjections of ANG-(1-12) (0.1–1.0 mM) into the RVLM elicited increases in mean arterial pressure (MAP) and heart rate (HR). Maximum cardiovascular responses were elicited by 0.5 mM ANG-(1-12); this concentration was used in other experiments described below. Microinjections of ANG-(1-12) increased greater splanchnic nerve activity (GSNA). The tachycardic responses to ANG-(1-12) were not altered by bilateral vagotomy. The cardiovascular responses elicited by ANG-(1-12) were attenuated by microinjections of an angiotensin II type 1 receptor (AT₁R) antagonist (losartan), but not AT₂R antagonist (PD123319), into the RVLM. Combined inhibition of angiotensin converting enzyme (ACE) and chymase in the RVLM abolished ANG-(1-12)-induced responses. ANG-(1-12)-immunoreactive cells were present in the RVLM. AT₁Rs and phenylethanolamine-N-methyl-transferase (PNMT) were present in the RVLM neurons retrogradely labeled by microinjections of Fluoro-Gold into the intermediolateral cell column of the thoracic spinal cord. ANG-(1-12)-containing neurons in the hypothalamic paraventricular nucleus did not project to the RVLM. These results indicated that: 1) microinjections of ANG-(1-12) into the RVLM elicited increases in MAP, HR, and GSNA, 2) both ACE and chymase were needed to convert ANG-(1-12) into angiotensin II, and 3) AT₁Rs, but not AT₂Rs, in the RVLM mediated ANG-(1-12)-induced responses.

Keywords

angiotensin II; blood pressure; captopril; chymostatin; heart rate; losartan; sympathetic nerve activity

Introduction

The rostral ventrolateral medullary pressor area (RVLM) is critical in the central regulation of cardiovascular function (Dampney et al., 2003; Guyenet, 2006; Sapru, 2002; Willette et al., 1983). Monosynaptic projections from the RVLM to the intermediolateral cell column (IML) of the thoraco-lumbar cord mediate the sympathetic outflow from the RVLM. Glutamate is the primary neurotransmitter in these bulbo-spinal projections (Morrison, 2003;

Sundaram & Sapru, 1991). Although the role of the RVLM in controlling cardiovascular functions is well established, information regarding the role of different putative neurotransmitters in this brain area in modulating these functions is still emerging.

Angiotensin II (ANG II) is one of the peptides implicated as a neurotransmitter or neuromodulator in the RVLM (Dampney et al., 2007). Although angiotensin II type 1 receptors (AT₁Rs) are not involved in the generation of resting sympathetic tone in the RVLM, these receptors have been implicated in the tonic sympathetic activity in spontaneously hypertensive rats (SHR), genetically modified rats in which either AT₁Rs are upregulated or endogenous levels of ANG II are increased, heart failure and salt-deprivation (Dampney et al., 2002). ANG II has also been implicated as a neurotransmitter in the projection from the hypothalamic paraventricular nucleus (PVN) to the RVLM (Tagawa & Dampney, 1999). AT₁Rs in the RVLM have been reported to mediate the pressor responses to aversive stress (Chen et al., 2012).

Recently a new angiotensin, angiotensin-(1-12) [ANG-(1-12)], has been identified (Nagata et al. 2006). In the rat, ANG-(1-12) has been reported to elicit a pressor response which was blocked by prior intravenous administration of an angiotensin converting enzyme (ACE) inhibitor or an AT₁R antagonist (Nagata et al. 2006). ANG-(1-12) has also been named proangiotensin-12 because its actions are mediated via rapid conversion to ANG II (Nagata et al. 2006).

Angiotensinogen is considered to be the substrate for generation of ANG II. Renin is not involved in the formation of ANG-(1-12) (Ferrario et al. 2009; Trask et al. 2008). It has been suggested that ANG-(1-12) may serve as a renin-independent alternate substrate for the immediate formation of ANG II in several organs (Trask et al. 2008). In the brain tissue the concentration of ANG-(1-12) is about five times greater than that of ANG II (Nagata et al. 2006). Several recent observations suggest that ANG-(1-12) may play a role in cardiovascular regulation. For example, cells immunoreactive for ANG-(1-12) have been identified in cardiovascular regulatory areas like the nucleus tractus solitarius (NTS) and PVN (Arnold et al. 2010; Chitravanshi et al., 2011), microinjections of ANG-(1-12) into the NTS and hypothalamic arcuate nucleus (ARC/N) and PVN of the rat have been reported to elicit depressor and pressor responses, respectively (Arakawa et al., 2011; Arnold et al. 2010; Chitravanshi & Sapru, 2011).

The focus of this investigation was to study the cardiovascular actions of ANG-(1-12) in the RVLM with the long-term goal of expanding our knowledge regarding the central cardiovascular actions of this new angiotensin. No information is available in the literature on the actions of ANG-(1-12) in the RVLM.

Methods

General procedures

Adult male Wistar rats (Charles River Laboratories, Wilmington, MA), weighing 320–350 g, were used in this study (n = 65). The animals were housed under controlled conditions with a 12:12-hr light-dark cycle. The animals had access to food and water ad libitum. The experiments were done according to the “Guide for the Care and Use of Laboratory Animals”, National Institutes of Health, (8th Edition, 2010) and with the approval of the Institutional Animal Care and Use Committee of this university.

We have previously published the details of the procedures used in this study (Arakawa et al., 2011; Chitravanshi & Sapru, 2011; Chitravanshi et al., 2011). Briefly, the rats were anesthetized with inhalation of isoflurane (2–3% in 100% oxygen). The trachea was

cannulated and the rats were artificially ventilated; the tidal volume and rate of respiration were adjusted so that end-tidal CO₂ remained at 3.5–4.5%. One of the veins was cannulated and urethane (1.2–1.4 g/kg) was injected intravenously in 8–9 aliquots at 2-min intervals. When the urethane administration was completed, isoflurane administration was terminated. The adequacy of anesthesia was indicated by the absence of a blood pressure (BP) response and/or withdrawal of the limb in response to pinching of a hind paw. Rectal temperature was maintained at 37.0 ± 0.5°C. Femoral arterial BP and heart rate (HR) were recorded by standard techniques. All of the tracings were stored on a computer hard drive using a data acquisition system obtained from Cambridge Electronic Design Ltd (CED), Cambridge, UK. At the end of the experiment, the rats were deeply anaesthetized with a high dose of urethane (2 g/kg, i.v.). An incision was made in one of the intercostal muscles to produce a pneumothorax and cessation of heart beat indicated that euthanasia was complete.

Bilateral vagotomy

The vagus nerves were identified bilaterally low in the neck and silk sutures were placed loosely around them. At the time bilateral vagotomy was needed, the silk sutures were pulled gently, one at a time, and the vagus nerves were sectioned. About 50–60 min were allowed for stabilization of baseline BP and HR after bilateral vagotomy.

Decerebration

Experiments were performed on unanesthetized decerebrate rats in order to exclude the effects of urethane, if any, on ANG-(1-12)-induced responses. The procedure for decerebration was similar to that reported by us earlier which included ligation of internal and external carotid and pterygopalantine arteries prior to the transection of the brain at mid-collicular level (Sapru & Krieger, 1978). After the decerebration procedure was completed, a stabilization period of 50–60 min was allowed.

Microinjections

The rats were placed in a prone position in a stereotaxic instrument with the bite bar 18 mm below the interaural line. The dorsal medulla was exposed and microinjections were made using multi-barreled glass-micropipettes (tip size 20–40 µm). Each barrel was connected to a channel on a picospritzer. Three barrels contained L-glutamate (L-GLU), artificial cerebrospinal fluid (aCSF), and ANG-(1-12). The remaining barrels contained either an AT₁R or AT₂R antagonist, or an ACE or chymase inhibitor. The micropipette was inserted into the brain perpendicularly for making microinjections. The RVLM sites eliciting pressor and tachycardic responses were identified by microinjections of L-GLU (5 mM). The coordinates for the RVLM were 2.0–2.2 mm rostral to the calamus scriptorius, 1.8–2.0 mm lateral to the midline, and 2.8–3.0 mm deep from the dorsal medullary surface. The volume of all microinjections into the RVLM was 100 nl; the selection of this volume was based on our previous studies (Kawabe et al., 2006). Unless indicated otherwise, all microinjections into the RVLM were unilateral. The volumes of microinjections were pressure ejected and visually confirmed by the displacement of fluid meniscus in the barrel containing the solution using a modified binocular horizontal microscope with a graduated reticule in one eye-piece. The duration of microinjection was 10 sec. Microinjections of aCSF (100 nl, pH 7.4) were used as controls.

Nerve recording

The greater splanchnic nerve (GSN) was selected for recording because it contains sympathetic fibers innervating major abdominal vascular beds and the kidney. A few mm of the central end of the nerve were desheathed and whole nerve activity was amplified (× 20,000–30,000), filtered (100–5000 Hz), digitized and stored on a computer hard drive. The

digitized signals were full wave rectified and integrated using Spike2 software (CED, UK). The baroreflex sensitivity of the GSN sympathetic nerve activity (GSNA) was indicated by its inhibition in response to the pressor responses elicited by bolus injections of phenylephrine (PE; 10 µg/kg. i.v.).

Drugs, chemicals and antibodies

The following drugs and chemicals were used: Angiotensin-(1-12), angiotensin II, buprenorphine hydrochloride, captopril (ACE inhibitor; Migdalof et al. 1984), cefazolin, chymostatin (chymase inhibitor; He et al. 1999), isoflurane, L-glutamate monosodium, L-phenylephrine hydrochloride (PE), losartan (selective AT₁R antagonist), pentobarbital sodium, PD123319 (AT₂R antagonist; Blankley et al. 1991) and urethane. All of the solutions for the microinjections were freshly prepared in aCSF (pH 7.4). The concentration of drugs refers to their salts where applicable. The sources of drugs were as follows: Angiotensin-(1-12) and angiotensin II (American Peptide Company Inc., Sunnyvale, CA, USA), buprenorphine (Hospira Inc, Lake Forest, IL, USA), cefazolin (Westward Pharmaceuticals Corp, Eatontown, NJ, USA), pentobarbital (Diamondback Drugs, Scottsdale, AZ, USA), captopril, chymostatin, losartan, L-glutamate monosodium, L-phenylephrine and urethane (Sigma-Aldrich Chemicals, St. Louis, MO, USA), PD123319 (Tocris-Cookson Inc. Ellisville, MO, USA) and isoflurane (Baxter Pharmaceutical Products, Deerfield, IL, USA). The selection of doses of angiotensin II, buprenorphine, cefazolin, pentobarbital, captopril, chymostatin, losartan, L-glutamate, phenylephrine, PD123319, and urethane were based on our previous reports (Arakawa et al., 2011; Chitravanshi & Sapru, 2011; Chitravanshi et al., 2011).

The sources of primary antibodies were as follows: 1) rabbit anti-AT₁R antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA; cat# sc-1173), 2) rabbit pro-angiotensin-12, ANG-(1-12) antibody (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA; cat# H-002-35), and 3) rabbit anti-ANG II antibody (Phoenix Pharmaceuticals Inc.; cat# H-002-12), and 4) rabbit anti-phenylethanolamine-N-methyl-transferase (PNMT) antibody (EMD Millipore, Billerica, MA, USA; cat# AB110). Goat anti-rabbit IgG conjugated with Cy3 (Amax = 550 nm, Emax = 570 nm) (Jackson Immunoresearch, Laboratories, West Grove, PA, USA; cat# 111-165-003) was used as the secondary antibody.

Fluoro-Gold (FG; Fluorochrome Inc., Denver, CO, USA) or Lumafluor (LF; Lumafluor Corp., Durham, NC, USA) were used as retrograde tracers; Lumafluor was also used to mark the microinjection sites.

Immunohistochemistry

The following experiments were done for immunohistochemical studies: 1) detection of AT₁Rs in the RVLM neurons retrogradely labeled by injections of FG into the IML, 2) detection of PNMT in the RVLM neurons retrogradely labeled by injections of FG into the IML, 3) identification of the RVLM cells containing ANG-(1-12), and 4) detection of ANG-(1-12) and ANG II in the PVN neurons retrogradely labeled by microinjections of FG into the RVLM.

The following primary antibodies were used for above-mentioned experiments: 1) rabbit anti-AT₁R antibody (dilution 1: 100), 2) rabbit pro-angiotensin-12 (ANG-(1-12) antibody (dilution 1: 300), 3) rabbit anti-ANG II antibody (dilution 1: 200), and 4) rabbit anti-PNMT antibody (dilution 1: 1000). Controls for immunostaining procedures consisted of omission of the primary antibody used for AT₁Rs, ANG-(1-12), ANG II and PNMT from the protocol (negative control) and pre-absorption of the primary antibodies for AT₁Rs, ANG-(1-12) and ANG II with control peptides.

For retrograde labeling studies, the surgery was done under aseptic conditions. The rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), fixed in a prone position in a stereotaxic instrument and FG (4 %) was microinjected on one side either into the IML at T2, T5 or T7 (5 nl) or RVLM (5 nl) using the coordinates mentioned earlier. Absorbable gelatin sponge (Surgifoam, Ethicon Inc., Somerville, NJ, USA) was placed on the opened spinal or medullary surface, and the muscles and skin over the wound were sutured. The rats were kept alive for 5 days. At the end of 5-day survival period, the rats were anesthetized again with pentobarbital, a small hole was drilled into the parietal bone on one side, colchicine (120 µg) was injected into the lateral ventricle and another 2-day survival period was allowed. During each survival period, an antibiotic (Cefazolin, 30 mg/kg) and an analgesic (Buprenorphine, 0.05 mg/kg) were administered subcutaneously twice a day for 2–3 days.

At the end of the 2nd survival period, the rats were deeply anaesthetized with urethane (2 g/kg, i.v.; administered in divided doses), perfused transcardially and fixed with 2% paraformaldehyde solution containing 0.2% picric acid, the brains were removed and fixed in 2% paraformaldehyde for 72–96 hrs. On completion of the fixation procedure, serial sections of the medulla or hypothalamus containing the retrogradely labeled neurons were cut (40 µm) in a vibratome (model 1000 Plus, The Vibratome Company, St. Louis, MO, USA), and placed in 24-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The sections were rinsed (rinsing of sections was always done 3 times for 10 min each time) with 0.1 M phosphate buffered saline (PBS), blocked for 1 hour at room temperature with normal goat serum containing 0.3% Triton X-100 in PBS (TPBS). The tissue culture plates containing the sections were then placed on a shaker and incubated at 4°C for 24 hours with the primary antibody against the peptide or receptor to be detected. Next, the sections were incubated at 4°C for 24 hrs with a secondary antibody conjugated with a fluorescent reporter molecule. On completion of incubation with the primary and secondary antibodies, the sections were rinsed in PBS, mounted on subbed slides, covered with Citifluor Mountant media (Ted Pella Inc., Redding, CA, USA) and coverslipped. The images of the sections were captured, 0.9 µm apart, by laser scanning confocal microscopy (AIR confocal microscope, Nikon Instruments Inc., Melville, NY, U.S.A.).

Histology

Microinjection sites were marked by microinjections of diluted (1: 42) green Lumafluor retrobeads. The animals were perfused and fixed with 4% paraformaldehyde, serial sections of the medulla were cut (30–40 µm), mounted on slides, covered with Citifluor Mountant media and coverslipped. The microinjection sites were identified, using a fluorescence microscope, photographed and compared with a standard atlas (Paxinos & Watson, 2007).

Statistical analyses

For comparison of MAP and HR responses, the mean and standard error of mean (SEM) were calculated for maximum changes in these values in response to microinjections of ANG-(1-12), ANG II, or L-GLU into the RVLM. In the concentration-response studies, comparisons of maximum increase in MAP and HR in different groups of rats were made by using a one-way ANOVA followed by Tukey-Kramer's multiple comparison. Desensitization to multiple injections of ANG-(1-12) was tested by repeated-measures ANOVA followed by Tukey-Kramer's multiple comparison. Student's paired *t*-test was used in experiments where each animal served as its control. Student's unpaired *t*-test was used to compare responses in different groups of rat. For the analysis of nerve activity, control value represented the average amplitude of integrated GSNA during 35 sec period before the intravenous (i.v.) administration of PE or the microinjections of drugs into the RVLM. The nerve activity remaining after sectioning the nerve rostrally at the end of the experiment was

considered to be the noise level which was subtracted from the whole GSNA. Maximum change in GSNA amplitude, induced by i.v. administration of PE or microinjections of drugs into the RVLM, was expressed as percent change from the baseline value of GSNA amplitude. The area under curve (AUC) of changes in GSNA (baseline activity subtracted) was also determined using Spike2 software (CED, UK) (Rahman et al., 2012). The mean values of the integrated nerve signals and AUC were compared using Student's paired *t*-test. In all cases, the differences were considered significant at $P < 0.05$.

Results

Baseline values for MAP and HR in urethane-anaesthetized rats were 89 ± 4 mmHg and 376 ± 6 bpm, respectively ($n = 48$).

Concentration-response of ANG-(1-12)

As mentioned in the Methods section, all microinjections into the RVLM were unilateral unless indicated otherwise, the volume of microinjections was 100 nl and the RVLM was identified by microinjections of L-GLU (5 mM) which stimulates neurons but not fibers of passage. Microinjections of L-GLU into the RVLM elicited pressor and tachycardic responses. The interval between the microinjections of L-GLU and other agents was at least 5 min. Microinjections of ANG-(1-12) (0.1–1.0 mM) into the RVLM elicited increases in MAP and HR. In each rat, only two concentrations of ANG-(1-12) were microinjected into the RVLM in a random fashion. The interval between the two microinjections of ANG-(1-12) was at least 45 min. The maximum pressor responses were elicited by microinjections of 1.0 mM concentration of ANG-(1-12) (Table 1). However, the differences between the responses elicited by 0.5 mM and 1.0 mM concentrations of ANG-(1-12) were not statistically different. Therefore, the smaller concentration (0.5 mM) was selected for other experiments. For comparison, the maximally effective concentration of ANG II was also microinjected into the RVLM. Microinjections of ANG II (0.5 mM) into the RVLM elicited cardiovascular responses similar to those of ANG-(1-12) (Table 1). The onset of cardiovascular responses to microinjections of ANG-(1-12) (0.5 mM) into the RVLM (16.6 ± 2.1 sec) was significantly ($P < 0.05$) longer than the onset of action (10.3 ± 1.3 sec) of microinjections of ANG II (0.5 mM) at the same site. The durations of cardiovascular responses to microinjections of ANG-(1-12) and ANG II (0.5 mM each) into the RVLM were 9.6 ± 1.8 and 8.2 ± 1.1 min, respectively. The peak effects of the same concentrations of these two peptides (2.6 ± 0.4 and 2.4 ± 0.2 min, respectively) were not significantly different ($P > 0.05$). Microinjections of aCSF (100 nl) elicited no responses.

Anesthesia did not alter the responses to ANG-(1-12). For example, microinjections of ANG-(1-12) (0.5 mM) into the RVLM of urethane-anesthetized and unanesthetized decerebrate rats elicited an increase of 10.6 ± 1.2 and 12.8 ± 1.1 mmHg, respectively; the difference in responses was not statistically significant ($P > 0.05$).

Site-specificity of ANG-(1-12)-induced responses

The ANG-(1-12)-induced pressor and tachycardic responses were site-specific because similar microinjections into the adjacent areas elicited opposite responses. For example, microinjections of ANG-(1-12) (0.5 mM) into an area about 1 mm caudal to the RVLM (i.e., 0.8–1.0 mm rostral to the calamus scriptorius, 1.8–2.0 mm lateral to the midline, and 2.6–3.0 mm deep from the dorsal medullary surface) elicited depressor and bradycardic responses ($n = 4$). This area corresponds to the caudal ventrolateral medullary depressor area (CVLM) (Marchenko & Sapru, 2003). Microinjections of L-GLU (0.5 mM) into the CVLM elicited a decrease in MAP (-24.3 ± 1.7 mmHg) and HR (-35.3 ± 8.1 beats/min). Microinjections of

ANG-(1-12) (0.5 mM) at the same site also elicited a decreases in MAP (-9.0 ± 0.8 mmHg) and HR (-12.5 ± 0.9 beats/min).

Reproducibility of ANG-(1-12)-induced responses

The increases in MAP in response to 3 consecutive microinjections of ANG-(1-12) (0.5 mM) at 45 min intervals were 10.8 ± 2.0 , 10.0 ± 1.5 , and 9.0 ± 0.8 mmHg, respectively and the increases in HR were 15.2 ± 2.6 , 13.4 ± 2.4 , and 15.8 ± 3.8 bpm, respectively ($n = 5$). A repeated measure ANOVA showed that these values were not statistically different ($P > 0.05$). Because no tachyphylaxis of responses was observed with repeated microinjections of ANG-(1-12) (0.5 mM) at 45 min intervals, the interval between different microinjections of ANG-(1-12) was at least 45 min in all experiments.

Effect of bilateral vagotomy on ANG-(1-12)-induced responses

Bilateral vagotomy did not alter increases in MAP and HR elicited by unilateral microinjections of either L-GLU (5 mM) or ANG-(1-12) (0.5 mM) into the RVLM (Table 2).

Effect of AT₁R blockade on ANG-(1-12)-induced responses

A tracing showing the effects of losartan (AT₁R antagonist) on ANG-(1-12)-induced cardiovascular responses in the RVLM is presented in Fig. 1. The RVLM site was identified by a microinjection of L-GLU (5 mM; Fig. 1A). After 2 min, microinjection of aCSF (100 nl) did not elicit a response (not shown). Twenty min later, microinjection of ANG-(1-12) (0.5 mM) elicited increases in HR, MAP and systolic and diastolic pressure (Fig. 1B). Forty five min later, losartan (10 mM) was microinjected into the RVLM; no changes in MAP and HR were elicited (Fig. 1C). Two minutes later, ANG-(1-12) (0.5 mM) was again microinjected at the same site; the effect of ANG-(1-12) was blocked (Fig. 1D). Five min later, microinjection of L-GLU (5 mM) continued to elicit the increase in HR, MAP and pulsatile arterial pressure (PAP) (Fig. 1E). The ANG-(1-12)-induced increases in MAP and HR did not recover to the initial values within 60 min after the microinjection of losartan (10 mM) into the RVLM. Group data for ANG-(1-12)-induced increases in MAP and HR before and after the microinjection of losartan are shown in Table 2.

Effect of AT₂R blockade on ANG-(1-12)-induced responses

Microinjections of a selective AT₂R antagonist (PD123319; 50 mM) into the RVLM did not alter the cardiovascular responses elicited by microinjections of ANG-(1-12) (0.5 mM) at the same site (Table 2).

Effect of ACE inhibition on ANG-(1-12)-induced responses

Microinjections of captopril (200 mM; an ACE inhibitor) into the RVLM significantly reduced the increases in MAP and HR induced by subsequent microinjections (within 2 min) of ANG-(1-12) (0.5 mM) at the same site (Table 2). ANG-(1-12)-induced increases in MAP and HR did not completely recover to the initial values within 60 min of the microinjection of captopril (200 mM) into the RVLM. Captopril did not alter the cardiovascular responses to microinjections of L-GLU into the RVLM (Table 2). Unilateral microinjections of captopril alone into the RVLM did not elicit any cardiovascular responses.

Effect of a chymase inhibition on ANG-(1-12)-induced responses

Microinjections of chymostatin (10 mM; a chymase inhibitor) into the RVLM significantly attenuated the increases in MAP and HR induced by subsequent microinjections (within 2 min) of ANG-(1-12) (0.5 mM) at the same site (Table 2). ANG-(1-12)-induced increases in MAP and HR did not completely recover to the initial values within 60 min of the

microinjection of chymostatin (10 mM) into the RVLM. Chymostatin did not alter the cardiovascular responses to microinjections of L-GLU into the RVLM (Table 2). Unilateral microinjections of chymostatin alone (10 mM) into the RVLM did not elicit any cardiovascular responses.

Effect of ACE and chymase inhibition on ANG-(1-12)-induced responses

Sequential microinjections of captopril (200 mM) and chymostatin (10 mM) elicited greater attenuation of ANG-(1-12)-induced cardiovascular responses when compared with the attenuation elicited by either captopril or chymostatin alone (Table 2). Combined microinjections of captopril and chymostatin into the RVLM on one side did not alter baseline MAP and HR.

Effect of microinjections of ANG-(1-12) on sympathetic nerve activity

Fig. 2 shows a typical recording of the effect of microinjections of ANG-(1-12) into the RVLM on efferent GSNA. A bolus injection of PE (10 µg/kg, i.v.) increased MAP which, in turn, elicited reflex bradycardia and inhibition of efferent GSNA (Fig. 2A). Fifteen min later, when the GSNA recovered to baseline, a microinjection of L-GLU (5 mM) into the RVLM elicited an increase in GSNA (Fig. 2B). After 20 min, microinjection of aCSF (100 nl) into the same RVLM site did not alter the GSNA (not shown). Two min later, microinjection of ANG-(1-12) (0.5 mM) into the RVLM increased efferent GSNA (Fig. 2C). Forty five min later, a combination of captopril (200 mM) and chymostatin (10 mM) was microinjected into the RVLM; no changes in GSNA were elicited (Fig. 2D). Two min later, ANG-(1-12) (0.5 mM) was again microinjected at the same site; the effect of ANG-(1-12) was blocked (Fig. 2E). Five min later, microinjection of L-GLU (5 mM) continued to elicit an increase in efferent GSNA (Fig. 2F).

Group data ($n = 5$) for maximum effects of ANG-(1-12) on GSNA are shown in Fig. 3. All changes in GSNA, expressed as percentages, refer to comparison with the basal nerve activity. Intravenous bolus injection of PE (10 µg/kg) decreased GSNA (Fig. 3A, a). Microinjection of L-GLU (5 mM) into the RVLM elicited significant increase in GSNA (Fig. 3A, b). Twenty min later, microinjection of ANG-(1-12) (0.5 mM) at the same site elicited significant increases in GSNA (Fig. 3A, c). Twenty min later, a combined solution of captopril (200 mM) and chymostatin (10 mM) was microinjected into the RVLM; no significant changes in GSNA were elicited (Fig. 3A, d). Two min after the blockade of ACE and chymase, microinjection of ANG-(1-12) at the same site elicited significantly smaller increases in GSNA (Fig. 3A, e). On the other hand, the blockade of ACE and chymase did not alter L-GLU-induced increase in GSNA (Fig. 3A, f). In the same group of rats, the effects of different pharmacological manipulations on the changes in the AUC of GSNA are presented in Fig. 3B. All changes in the AUC of GSNA are expressed as percentages and refer to comparison with the basal nerve activity. Intravenous bolus injection of PE (10 µg/kg) decreased the AUC of GSNA (Fig. 3B, a). Microinjection of L-GLU (5 mM) into the RVLM elicited a significant increase in the AUC of GSNA (Fig. 3B, b). Twenty min later, microinjection of ANG-(1-12) (0.5 mM) at the same site elicited a significant increase in the AUC of GSNA (Fig. 3B, c). After an interval of 20 min, combined microinjections of captopril (200 mM) and chymostatin (10 mM) into the RVLM did not alter the AUC of GSNA (Fig. 3B, d). Two min after the blockade of ACE and chymase, microinjection of ANG-(1-12) at the same site elicited a significantly smaller increase in the AUC of GSNA (Fig. 3B, e) while L-GLU-induced increase in the AUC of GSNA was not altered (Fig. 3B, f).

Neurons immunostaining for AT₁Rs, ANG-(1-12) and PNMT in the RVLM

The presence of neurons immunoreactive to AT₁Rs, ANG-(1-12) and PNMT in the RVLM was determined in 12 rats. Fig. 4A shows retrogradely labeled neurons in the RVLM after microinjections (5 nl) of FG in the IML at T2–T7 levels on one side. Neurons immunostaining for AT₁Rs were also observed in this region (Fig. 4B). A merged image of FG labeled and AT₁R immunostaining neurons in the same section are shown in Fig. 4C; AT₁Rs were present in the RVLM neurons retrogradely labeled from the IML. Specificity of the primary antibody for AT₁Rs was indicated by lack of staining for these proteins when the primary antibody was either omitted from the protocol (Fig. 4D) or pre-absorbed by incubation with the control peptide (Fig. 4E).

Fig. 5A shows retrogradely labeled neurons in the RVLM after microinjections of FG (5 nl) in the IML at T2–T7 levels. Neurons immunostaining for PNMT were also observed in this region (Fig. 5B). A merged image of FG labeled and PNMT immunostaining neurons in the same section are shown in Fig. 5C; PNMT was present in the RVLM neurons retrogradely labeled from the IML. Specificity of the primary antibody for PNMT was indicated by lack of staining for this enzyme when the primary antibody was omitted from the protocol (Fig. 5D).

Fig. 6A shows neurons immunostaining for ANG-(1-12) in the RVLM. Specificity of the primary antibody for ANG-(1-12) was indicated by lack of staining for this peptide when the primary antibody was omitted from the protocol (Fig. 6B) and when the primary antibody was pre-absorbed with control peptide (Fig. 6C). We have previously reported that the antibody used for ANG-(1-12) did not cross react with ANG II because immunostaining for ANG-(1-12) persisted even after the pre-absorption of the antibody with ANG II peptide (Chitravanshi et al., 2011).

Neurons immunostaining for ANG-(1-12) and ANG II in the PVN

Immunohistochemical studies were also done to determine if PVN neurons projecting to the RVLM contained ANG-(1-12). Fig. 7A shows retrogradely labeled neurons in the caudal PVN (1.8 mm caudal to the bregma) after microinjections of FG (5 nl) in the RVLM on one side. Neurons immunostaining for ANG-(1-12) were observed in the caudal PVN (Fig. 7B). A merged image of FG labeled and ANG-(1-12) immunostaining neurons in the same sections is shown in Fig. 7C; the FG-labeled neurons and ANG-(1-12) containing neurons did not merge indicating that ANG-(1-12) containing neurons in the caudal PVN do not project to the RVLM.

Fig. 8A shows retrogradely labeled neurons in the caudal PVN (1.8 mm caudal to the bregma) after microinjections of FG (5 nl) in the RVLM on one side. Neurons immunostaining for ANG II were observed in the caudal PVN (Fig. 8B). A merged image of FG labeled and ANG II immunostaining neurons in the same sections is shown in Fig. 8C; the FG-labeled neurons and ANG II containing neurons merged indicating that ANG II containing neurons in the caudal PVN project to the RVLM. Specificity of the primary antibody for ANG II was indicated by lack of staining for this peptide when the primary antibody was either omitted from the protocol (Fig. 8D) or pre-absorbed by incubation with the control peptide (Fig. 8E).

Histological identification of microinjection sites

A typical RVLM microinjection site marked with Lumafluor (100 nl) is shown in Fig. 9A. Figures 9B–9C represent composite diagrams of coronal sections of RVLM showing microinjection sites (n = 10). In each of these figures, each dark circle represents the centre of one microinjection site in one animal. The RVLM microinjection sites were located 2.0–

2.1 mm rostral to the calamus scriptorius, 1.8–2.0 mm lateral to the midline and 2.8–3.2 mm deep from the medullary surface.

Discussion

The major findings of the study were as follows: 1) Microinjections of ANG-(1-12) into the RVLM elicited increases in MAP, HR, and efferent GSNA, 2) these effects were mediated via activation of AT₁Rs, but not AT₂Rs, in the RVLM, 3) ANG-(1-12)-induced cardiovascular responses were attenuated by prior microinjections of either captopril (ACE inhibitor) or chymostatin (chymase inhibitor) into the RVLM, 4) combination of microinjections of captopril and chymostatin into the RVLM elicited a greater attenuation of the cardiovascular effects of ANG-(1-12), 5) RVLM neurons that were retrogradely labeled by microinjections of a tracer into the IML contained AT₁Rs, 6) ANG-(1-12) and PNMT containing neurons were present in the RVLM, 7) ANG-(1-12) was also present in the caudal PVN neurons but these neurons did not project to the RVLM, and 8) ANG II was also present in the caudal PVN neurons and these neurons did project to the RVLM.

It has been reported that ANG-(1-12) is converted into ANG II in the myocardial tissue and plasma (Nagata et al. 2006; Prosser et al. 2009; Trask et al. 2008). Conversion of ANG-(1-12) to ANG II has also been reported in the central nervous system (CNS), especially in the NTS, ARCn and PVN in the hypothalamus (Arakawa et al., 2011; Arnold et al. 2010; Chitravanshi & Sapru, 2011; Chitravanshi et al., 2011). The present study shows that the responses of ANG-(1-12) are mediated via its conversion to ANG II in the RVLM also. ACE is partly involved in the conversion of ANG-(1-12) to ANG II in these brain regions because microinjections of captopril attenuated ANG-(1-12)-induced responses. Chymase has been reported to be one of the alternate pathways by which ANG II is formed in various tissues (Prosser et al. 2009). ANG II formation has also been reported in the pituitary stalk and in the pineal gland (Baltatu et al., 1997). We have previously reported that chymase may be partially involved in the conversion of ANG-(1-12) to ANG II in the ARCn, NTS and PVN (Arakawa et al. 2011; Chitravanshi & Sapru, 2011; Chitravanshi et al., 2011). In this study we have demonstrated that in the RVLM also chymase may be partially involved in the conversion of ANG-(1-12) to ANG II because microinjections of chymostatin attenuated ANG-(1-12)-induced cardiovascular responses. Greater attenuation of ANG-(1-12)-induced cardiovascular responses was observed when both ACE and chymase were inhibited simultaneously in the RVLM. Thus, cardiovascular effects elicited by ANG-(1-12) in the RVLM are mediated via its conversion to ANG II which has been reported to elicit increases in MAP and HR when microinjected into the RVLM area (Allen et al., 1988; Muratani et al., 1991; Punnen et al., 1984). Topical application of ANG II to a restricted area of the ventral medullary surface, known as glycine sensitive area, has also been reported to result in an increase in BP (Andreatta et al., 1988). We have previously reported that combined microinjections of captopril and chymostatin attenuated the cardiovascular responses to ANG-(1-12) but not ANG II in the PVN (Chitravanshi et al., 2011). Like in the NTS, ARCn and PVN, the onset and peak effect of ANG-(1-12) in the RVLM was delayed when compared to that of ANG II which is consistent with the notion that ANG-(1-12) needs to be converted to ANG II before it elicits its actions.

Microinjections of losartan blocked the effects of ANG-(1-12) in the RVLM indicating that the responses to ANG-(1-12) were mediated via AT₁Rs. The blockade of ANG-(1-12)-induced responses could not be attributed to desensitization because the interval between the two doses of ANG-(1-12) was at least 45 min (desensitization to repeated injections of this angiotensin did not occur at this time interval). This result is consistent with other reports indicating that the cardiovascular effects of ANG-(1-12) are mediated via AT₁Rs (Arakawa et al. 2011; Arnold et al. 2010; Chitravanshi & Sapru, 2011; Chitravanshi et al., 2011;

Nagata et al. 2006; Seyedabadi et al., 2001). The activation of AT₁Rs can be attributed to ANG II generated from ANG-(1-12). AT₂Rs in the RVLM were not involved in ANG-(1-12)-induced cardiovascular responses because microinjections of PD123319 into the RVLM did not alter cardiovascular responses elicited by microinjections of ANG-(1-12) in the RVLM. In this study we have demonstrated that AT₁Rs are present on the RVLM neurons that were retrogradely labeled by microinjecting FG into the IML at T2–T7 level. These neurons may mediate pressor and tachycardic responses in yet unidentified situations when ANG-(1-12) is converted into ANG II and released in the RVLM.

Although microinjections of L-GLU, ANG-(1-12) and ANG II into the RVLM elicited pressor responses, the mechanisms by which their responses are mediated may be different. For example, in spontaneously hypertensive rats (SHR), bilateral microinjections of wortmannin, a phosphatidylinositol-3 kinase (PI3K) inhibitor, into the RVLM reduced the ANG II response to levels seen in Wistar Kyoto rat (WKY; control for SHR). Combined microinjections of the mitogen-activated protein kinase (MAPK) inhibitor (PD098059) and wortmannin into the RVLM abolished the responses to ANG II in both SHR and WKY (Seyedabadi et al., 2001). Thus, ANG II-induced response in SHR is mediated by both PI3K- and MAPK-dependent pathways in the RVLM while only PI3K-dependent pathway is needed in WKY for this response (Seyedabadi et al., 2001; Yang et al, 1996; Yang and Raizada, 1999). The role of MAPK and PI3K-dependent pathways in mediating ANG-(1-12) responses remains to be elucidated. In this context, it is interesting to note that MAPK and PI3K inhibitors did not alter the responses to microinjections of L-GLU into the RVLM (Seyedabadi et al., 2001). Increases in MAP, HR and GSNA elicited by microinjections of ANG-(1-12) into the RVLM were smaller compared to these responses elicited by microinjections of L-GLU at the same site. The differences between the effects of ANG-(1-12) and L-GLU could be due to the differences in the densities of AT₁ and glutamate receptors in the RVLM and/or due to differences in the mechanisms by which the responses to these two agonists are mediated (Seyedabadi et al., 2001”).

ANG-(1-12) containing neurons were found to be present in the RVLM. The presence of PNMT-containing neurons retrogradely labeled with FG microinjections into the IML indicated that the region where ANG-(1-12) containing neurons were found was RVLM. In this context, it may be pointed out that studies combining immunohistochemistry with in-situ hybridization have revealed the presence of PNMT containing neurons in the RVLM (Pilowsky et al., 2009). The ANG-(1-12) containing neurons in the RVLM may be activated under yet unidentified situations to release ANG-(1-12). Alternatively, NTS neurons containing ANG-(1-12) may be the source of ANG-(1-12) in the RVLM (Arnold et al. 2010). We have previously reported the presence of ANG-(1-12)-immunoreactive cells and fibres in the PVN (Chitravanshi et al., 2011). However, these neurons may not be the source of ANG-(1-12) in the RVLM because in the present study we have demonstrated that ANG-(1-12) containing neurons in the PVN did not project to the RVLM. However, we observed that ANG II containing neurons in the PVN did project to the RVLM. This observation is consistent with the notion that cardiovascular responses may be mediated by ANG II released at the terminals of direct projections of the PVN to the RVLM (Tagawa & Dampney, 1999). The situations when these PVN projections to the RVLM are activated remain to be identified. The specificity of the antibodies used in this study was established by lack of staining when the primary antibody was omitted in the protocol or when it was pre-incubated with a control peptide. We have previously established that the antibody used for ANG-(1-12) did not cross react with ANG II (Chitravanshi et al., 2011).

Based on current knowledge regarding the role of RVLM in cardiovascular regulation (Dampney et al., 2003; Guyenet, 2006; Sapru, 2002), the mechanism of increases in MAP, HR, and GSNA elicited by microinjections of ANG-(1-12) into the RVLM can be explained

as follows. Microinjections of ANG-(1-12) into the RVLM result in the generation of ANG II which may excite the RVLM neurons involved in cardiovascular regulation via AT₁Rs. We have previously shown that extracellularly recorded activity of some neurons (e.g. barosensitive NTS neurons) is increased after direct application of ANG-(1-12) to these neurons and this effect is blocked by prior application of captopril and chymostatin indicating that the effect is mediated via formation of ANG II (Chitravanshi & Sapru, 2011). The mechanism of neuronal excitation elicited by ANG-(1-12) is not clear at this time. However, ANG II has been reported to excite neurons in the RVLM (Li & Guyenet, 1995; Oshima et al., 2008). ANG II derived from ANG-(1-12) may stimulate RVLM neurons via mechanisms established in the RVLM and other brain areas. These mechanisms include pre- and/or post-synaptic mechanisms, attenuation of synaptic GABA release (Li et al. 2005), presynaptic disinhibition (Li et al. 2003) and reduction in resting K⁺ conductance of neurons (Li & Guyenet, 1996).

Conclusion

In the CNS, there is a wide distribution of angiotensinogen and AT₁Rs but renin levels are low (Grobe et al. 2008). This mismatch has created doubts regarding the synthesis of ANG II from angiotensinogen in the CNS. Another explanation for the possibility of the absence of renin-angiotensin-system (RAS) in the CNS is that the presence of a receptor in a particular brain area does not necessarily imply the presence of a relevant/expected neurotransmitter in that region. Nevertheless, the discovery of ANG-(1-12) has prompted the speculation that this peptide may provide an alternate renin-independent pathway for the formation of ANG II in the CNS and it may serve as a precursor for ANG II in the central pathways regulating cardiovascular function. In this context, it may be noted that renin is not involved in the formation of ANG-(1-12) from angiotensinogen (Ferrario et al. 2009). The results of the present study in the RVLM support the role of ANG-(1-12) as a precursor for ANG II. Thus, ANG-(1-12) is converted to ANG II in the RVLM, which, in turn, elicits pressor and tachycardic responses via activation of AT₁Rs in this nucleus. Both ACE and chymase are involved in the conversion of ANG-(1-12) into ANG II in the RVLM. ANG-(1-12) containing cells were found to be present in the RVLM. These cells may be the source of endogenous ANG-(1-12) in the RVLM. In terms of the significance of the effects of ANG-(1-12) in the RVLM, it may be noted that up-regulation of the RAS in the brain has been reported in the experimental and genetic models of hypertension and heart failure (Morimoto et al. 2001; Yongue et al. 1991; Zheng et al. 2009). Signaling across blood brain barrier by ANG II has also been implicated in neurogenic hypertension (Paton et al. 2008; Tan et al. 2007). Central immunoneutralization of ANG-(1-12) has been reported to lower BP and improve baroreflex sensitivity and HR variability in hypertensive (mRen2)27 rats, indicating that endogenous ANG-(1-12), probably via ANG II formation, may play a role in central pathways controlling BP in hypertension (Isa et al. 2009). The present study provides the ground work for future investigations on the role of ANG-(1-12) in the RVLM in different pathological states such as heart failure and hypertension.

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Figure 1.

Blockade of ANG-(1-12) responses by losartan. Top trace: HR (beats/min). Middle trace: MAP (mmHg), Bottom trace: PAP (pulsatile arterial pressure; mmHg). A: the RVLM site was identified by a microinjection of L-GLU. B: 20 min later, microinjection of ANG-(1-12) at the same site elicited increases in HR, MAP and PAP. C: 45 min after microinjection of ANG-(1-12), losartan (10 mM) was microinjected at the same site; no responses in HR, MAP and PAP were elicited. D: within 2 min, ANG-(1-12) was again microinjected at the same site; the responses to ANG-(1-12) were abolished. E: microinjection of L-GLU, 5 min later, elicited the usual increases in HR, MAP and PAP. The volume of all microinjections was 100 nl. The concentrations of L-GLU and ANG-(1-12) were 5 mM and 0.5 mM, respectively.

Figure 2.

Effect of ANG-(1-12) on sympathetic nerve activity. Top trace: MAP (mmHg), 2nd trace: PAP (mmHg), 3rd trace: integrated GSNA ($\int$ GSNA, μ V/1ms), and bottom trace: whole GSNA (μ V). A: Reflex inhibition of GSNA elicited by pressor response induced by phenylephrine (PE, 10 μ g/kg, i.v.) indicated that the GSNA was barosensitive. B: 15 min later, microinjection of L-GLU into the RVLM increased MAP, PAP, integrated GSNA and whole GSNA. C: Twenty min later, microinjection of ANG-(1-12) into the RVLM increased the MAP, PAP, integrated GSNA and whole GSNA. D: Forty five min later, microinjection of captopril (200 mM) and chymostatin (10 mM) into the RVLM did not elicit any response. E: Two min later, microinjection of ANG-(1-12) at the same site failed to elicit the responses. F: 5 min later, microinjection of L-GLU continued to elicit usual cardiovascular responses. The volume of all microinjections was 100 nl. The concentrations of L-GLU and ANG-(1-12) were 5 mM and 0.5 mM, respectively.

Figure 3.

A: Group data showing ANG-(1-12)-induced changes in the GSNA (n = 5). a: Intravenous bolus injection of PE (10 µg/kg) significantly decreased GSNA ($-92.3 \pm 12.1\%$; $P < 0.01$). b: 15 min later, microinjections of L-GLU into the RVLM elicited significant ($P < 0.01$) increases in GSNA ($72.7 \pm 7.5\%$). c: Twenty min later, microinjection of ANG-(1-12) at the same site elicited significant ($P < 0.01$) increases in the GSNA ($23.7 \pm 3.3\%$). d: After 45 min, microinjection of captopril (200 mM) and chymostatin (10 mM) at the same site did not elicit significant changes in GSNA. e: Two min after the microinjection of captopril and chymostatin into the RVLM, the increase in GSNA ($5.9 \pm 1.1\%$) induced by ANG-(1-12) was significantly attenuated (compare with c; $**P < 0.01$). f: Two min later, L-GLU microinjected into the RVLM elicited significant increase ($67.9 \pm 3.3\%$, $P < 0.01$) in the GSNA that was not significantly different ($P > 0.05$) from b. B: In the same group of rats, data showing ANG-(1-12)-induced changes in area under curve (AUC) of GSNA. a: PE (10 µg/kg, i.v.) significantly decreased the AUC of GSNA ($-40.3 \pm 6.1\%$; $P < 0.01$). b: 15 min later, microinjections of L-GLU into the RVLM elicited significant ($P < 0.01$) increases in the AUC of GSNA ($31.2 \pm 6.1\%$). c: Twenty min later, microinjection of ANG-(1-12) at the same site elicited significant ($P < 0.01$) increases in the AUC of GSNA ($15.2 \pm 2.1\%$). d: After 45 min, microinjection of captopril (200 mM) and chymostatin (10 mM) at the same site did not elicit significant changes in the AUC of GSNA. e: Two min after the microinjection of captopril and chymostatin into the RVLM, the increase in the AUC of GSNA ($2.1 \pm 1.4\%$) induced by ANG-(1-12) was significantly attenuated (compare with c; $**P < 0.01$). f: Two min later, L-GLU microinjected into the RVLM elicited significant increase ($31.0 \pm 5.1\%$, $P < 0.01$) in the AUC of GSNA that was not significantly different ($P > 0.05$) from b. The volume of all microinjections was 100 nl. The concentrations of L-GLU and ANG-(1-12) were 5 mM and 0.5 mM, respectively.

Figure 4.

AT₁R-immunoreactive cells in the RVLM. A: RVLM neurons retrogradely labeled by microinjections (5 nl) of Fluoro-Gold (FG; 4%) in the ipsilateral IML at T7 (green fluorescence). B: AT₁R labeled cells in the RVLM (same section) (red fluorescence). C: Merged image (yellow fluorescence) indicates that AT₁R is present in neurons retrogradely labeled with FG; the neurons that show presence of FG and AT₁R are identified by white arrows in each panel. D: No staining for AT₁R was observed when the primary antibody was omitted from the protocol. E: Staining for AT₁R was absent when the primary antibody was pre-incubated with control peptide for AT₁R. All images were captured by confocal microscopy. AT₁R: angiotensin II type 1 receptor; IML: intermediolateral cell column; RVLM: rostral ventrolateral medullary pressor area. Bar in panel A = 200 μ m; magnification was identical in all panels.

Figure 5.

PNMT-immunoreactive cells in the RVLM. A: RVLM neurons retrogradely labeled by microinjections (5 nl) of Fluoro-Gold (FG; 4%) in the ipsilateral IML at T7 (green fluorescence). B: PNMT labeled cells in the RVLM (same section) (red fluorescence). C: Merged image (yellow fluorescence) indicates that PNMT is present in some neurons retrogradely labeled with FG; the neuron that shows presence of FG and PNMT is identified by white arrows in each panel. D: No staining for PNMT was observed when the primary antibody was omitted from the protocol. All images were captured by confocal microscopy. PNMT: phenylethanolamine-N-methyl transferase; IML: intermediolateral cell column; RVLM: rostral ventrolateral medullary pressor area. Bar in panel A = 50 μ m; magnification was identical in all panels.

Figure 6.

ANG-(1-12)-immunoreactive cells in the RVLM. A: ANG-(1-12) labeled cells in the RVLM (red fluorescence). B: No staining for ANG-(1-12) was observed when the primary antibody was omitted from the protocol. C: Staining for ANG-(1-12) was absent when the primary antibody was pre-incubated with control peptide for ANG-(1-12). All images were captured by confocal microscopy. ANG-(1-12): angiotensin-(1-12); RVLM: rostral ventrolateral medullary pressor area. Bar in panel A = 100 μ m; magnification was identical in all panels.

Figure 7.

ANG-(1-12)-immunoreactive cells in the PVN do not project to the RVLM. A: PVN neurons retrogradely labeled by microinjections (5 nl) of Fluoro-Gold (FG; 4%) in the ipsilateral RVLM (green fluorescence). B: ANG-(1-12) labeled cells in the PVN (same section) (red fluorescence). C: Merged images in panels A and B; there was no overlap of FG and ANG-(1-12) containing cells in the PVN indicating that ANG-(1-12)-containing cells in the PVN do not project to the RVLM. All images were captured by confocal microscopy. ANG-(1-12): angiotensin-(1-12); PVN: hypothalamic paraventricular nucleus; RVLM: rostral ventrolateral medullary pressor area; 3V: third ventricle. Bar in panel A = 200 μ m; magnification was identical in all panels.

Figure 8.

ANG II-immunoreactive cells in the PVN project to the RVLM. A: PVN neurons retrogradely labeled by microinjections (5 nl) of Fluoro-Gold (FG; 4%) in the ipsilateral RVLM (green fluorescence). B: ANG II labeled cells in the PVN (same section) (red fluorescence). C: Merged image (yellow fluorescence) indicates that ANG II is present in neurons retrogradely labeled with FG; the neurons that show presence of FG and ANG II are identified by white arrows in each panel. D: No staining for AT₁Rs was observed when the primary antibody was omitted from the protocol. E: Staining for AT₁Rs was absent when the primary antibody was pre-incubated with control peptide for AT₁Rs. Panels F, G and H show high magnifications of boxed areas in panels A, B and C, respectively. All images were captured by confocal microscopy. ANG II: angiotensin II; PVN: hypothalamic paraventricular nucleus; RVLM: rostral ventrolateral medullary pressor area; 3V: third ventricle. Bar in panel A = 200 μ m; magnifications in panels B, C, D and E are identical. Bar in panel F = 200 μ m; magnifications in panels G and H are identical.

Figure 9.

Histological identification of microinjection sites. A: A coronal section showing the microinjection site in the RVLM marked with Lumaflour (100 nl; arrow); the center of the site was located 2.0 mm rostral to the calamus scriptorius, 2.0 mm lateral to midline and 3.0 mm deep from the medullary surface. B and C: Composite drawings of sections showing the microinjection sites in the RVLM (2.0 and 2.1 mm rostral to the calamus scriptorius; $n = 10$). Amb: nucleus ambiguus; Py: pyramids; RVLM: rostral ventrolateral medullary pressor area; Sp5: spinal trigeminal tract.

Table 1

Concentration responses of microinjections of ANG-(1-12) into the RVLM

Concentration of ANG-(1-12)	Increase in MAP (mmHg)	Increase in HR (beats/min)
0.1 mM	3.2 ± 1.0	4.8 ± 1.9
0.2 mM	5.6 ± 0.2	10.0 ± 1.8
0.5 mM	10.6 ± 1.2 ^{a,e}	12.4 ± 1.2 ^c
1.0 mM	11.4 ± 1.8 ^{b,f}	12.8 ± 2.3 ^d
Concentration of ANG II	Increase in MAP (mmHg)	Increase in HR (beats/min)
0.5 mM	12.8 ± 0.7	16.6 ± 1.1

Values are means ± SEM; n = 5 each concentration. MAP, mean arterial pressure; HR, heart rate.

The differences in pressor and tachycardic responses elicited by 0.5 and 1 mM concentrations were not significant ($P > 0.05$).

The differences in pressor and tachycardic responses elicited by ANG-(1-12) (0.5 mM) and ANG II (0.5 mM) were not significant ($P > 0.05$).

^{a,b} The pressor responses were significantly greater than those elicited by 0.1 mM concentrations ($P < 0.01$).

^{c,d} The tachycardic responses were significantly greater than those elicited by 0.1 mM concentrations ($P < 0.05$).

^{e,f} The pressor responses were significantly greater than those elicited by 0.2 mM concentrations ($P < 0.05$).

Table 2
Effect of different procedures or antagonists on the responses induced by microinjections of ANG-(1-12) into the RVLM

Procedure or Injection of Antagonist Into the RVLM	ANG-(1-12)-Induced Increase in MAP (mmHg)		ANG-(1-12)-Induced Increase in HR (beats/min)		L-GLU-Induced Increase in MAP (mmHg)		L-GLU-Induced Increase in HR (beats/min)	
	Before	After	Before	After	Before	After	Before	After
Vagotomy	12.3 ± 3.8	11.7 ± 2.7	12.3 ± 3.9	11.3 ± 4.4	25.3 ± 6.4	22.3 ± 3.8	24.0 ± 7.8	19.7 ± 6.1
Losartan	11.4 ± 1.0	2.8 ± 0.6 [‡]	15.0 ± 2.0	3.4 ± 0.5 [‡]	19.0 ± 1.0	16.6 ± 1.2	33.8 ± 5.6	30.4 ± 3.0
PD-123319	11.0 ± 1.4	10.2 ± 1.5	14.6 ± 2.8	15.8 ± 3.5	19.8 ± 3.2	17.4 ± 2.7	28.8 ± 5.3	28.0 ± 5.8
Captopril	10.6 ± 0.5	3.4 ± 0.8 [‡]	18.8 ± 2.0	6.6 ± 1.2 [‡]	18.2 ± 1.1	17.6 ± 1.5	23.8 ± 1.2	19.2 ± 1.8
Chymostatin	10.4 ± 1.9	8.4 ± 1.6 [*]	16.6 ± 2.2	13.0 ± 1.6 [*]	18.6 ± 1.8	18.0 ± 1.4	33.2 ± 6.1	30.8 ± 5.8
Captopril + Chymostatin	11.2 ± 1.7	2.4 ± 0.7 [‡]	17.0 ± 5.2	4.4 ± 1.7 [‡]	20.2 ± 1.9	20.8 ± 1.8	26.2 ± 5.0	21.8 ± 4.6

Values are means ± SEM. The following concentrations of drugs were used: ANG-(1-12), 0.5 mM; L-glutamate (L-GLU), 5 mM; Losartan, 10 mM; PD-123319, 50 mM; Captopril, 200mM; Chymostatin, 10 mM. ANG-(1-12)-induced responses were smaller compared to the responses before the procedure or microinjection of antagonist; n = 5 in all groups except vagotomy where n = 4.

^{*} P < 0.05,

[‡] P < 0.01 and

[‡] P < 0.001.